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Polymerase Chain Reaction and Qβ Replicase Amplification

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The polymerase chain reaction (PCR) and Qβ replicase are two methods in which nucleic acid polymerases are used for amplification. Although these approaches share many similar problems concerning target contamination and probe specificity, they differ dramatically in their mechanisms of action and modes of application. The PCR method amplifies target sequences between two priming oligonucleotides and in essence amplifies a portion of the analyte. Qβ replicase, on the other hand, amplifies a specific template molecule hybridized to target sequences and therefore amplifies a signal component of the system. For this reason, Qβ replicase amplification has applications in areas other than for the detection of nucleic acid sequences. The requirements for application and the advantages of both PCR and Qβ replicase amplification are reviewed.

Nucleic acid polymerases are used by both the polymerase chain reaction (PCR) and Qβ replicase methods to amplify small quantities of DNA and RNA, respectively. The ability of these methods to detect only a few molecules of a nucleic acid target may permit their widespread application in areas only now being explored. Already PCR is routinely used in the field of molecular biology, and its use in clinical biology is becoming more common. Qβ replicase offers the additional ability to easily quantify the amount of target nucleic acid. Both methods are exquisitely sensitive and promise to become extremely important techniques in research and medicine.

Polymerase Chain Reaction

PCR is a three-step process that duplicates a DNA fragment bounded by two oligonucleotide primers (*1*). Each primer is complementary to one strand of the double-stranded DNA template used. The first step of the process is initiated by denaturing or separating the double-stranded DNA target into its single-stranded components by heating the sample to 90–95 °C in the presence of a large excess of the two primers. In the second step, the temperature of the reaction is lowered to about 10 °C below the melting temperature of the primers, and the primers are allowed to anneal or hybridize to their complementary sequence on the DNA template molecule. The third step of the reaction is carried out by raising the temperature of the reaction to 70–73 °C, the optimal temperature for extension of the primers by a DNA polymerase. One cycle of denaturation, annealing, and primer extension results in a doubling of the DNA target sequence. By repeating the number of cycles as many as 25–30 times, the DNA sequence flanking the primers can be amplified as much as 10^6-fold (*2*). The amount of amplification can be estimated by the formula $y = S(1 + e)^n$, where e represents the cycle efficiency of the process, n the number of cycles, S the initial number of targets, and y the number of DNA copies after amplification (*3*). PCR can also be used to assay RNA in a sample if the RNA template is first transcribed into DNA by reverse transcriptase (*4*, *5*).

Because each step of the PCR process is controlled by temperature, all of the required components can be added at once in a single reaction tube. The temperature of the reaction can be controlled manually or regulated automatically by one of the thermal cyclers specifically designed for the application. Reaction tubes with thin walls have also been designed to fit these thermal cyclers to improve thermal regulation.

PCR was originally carried out with the Klenow fragment of *Escherichia coli* DNA polymerase I. Because this enzyme was inactivated at the temperature used to denature the double-stranded DNA synthesized in the PCR process, addition of fresh Klenow fragment was required after each denaturation step. This "problem" was overcome by the introduction of a thermostable DNA polymerase obtained from the organism *Thermus aquaticus* ("Taq") (*2*).

Taq polymerase is not inactivated by incubation at temperatures of up to 95 °C and does not become limiting in the protocol until 25–30 cycles have been completed. At that time, the original DNA strands have been amplified ~10^6-fold. Greater amplification can be accomplished by diluting the amplified DNA 10^3- to 10^4-fold and subjecting it to a second series of 25–30 cycles of PCR in the presence of fresh Taq polymerase. As many as 10^9 to 10^{10} copies of DNA can be obtained this way. During the second series of PCR cycles, it is common to use a different set of primers (nested), which hybridize downstream from the original primers. The use of nested primer sets not only increases the sensitivity of PCR but also can potentially enhance its specificity (*5*, *6*). A disadvantage of using nested primers is the potential for contamination during transfer of the sample from the first PCR reaction mixture to the second.

The oligonucleotide primers used in PCR are usually designed on the basis of the known DNA sequence of the DNA target as well as on the knowledge of similar DNA sequences that may be present in the sample. Hybridization of the primer to more than one site can result in fragments of various sizes and composition. Primers are typically 16–24 nucleotides long with a guanine-cy-

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tosine content of 50%. Primer optimization and selection is an empirical process. Each primer pair must be evaluated for sensitivity and specificity by use with actual samples.

The number of nucleotides separating the two primers determines the size of the predominant DNA fragment(s) produced during PCR. Fragments as long as 2 kb can be generated (*7*). For identification of microorganisms, fragments of 100–750 bases are routinely synthesized. Size analysis of the DNA fragments produced during PCR is frequently incorporated into detection schemes for this procedure. At the completion of PCR, aliquots of the reaction mixture are commonly electrophoresed on agarose gels and made visible by using an intercalating dye such as ethidium bromide (*7*). The addition of size markers to the gel allows identification of appropriate length fragments.

That a fragment of the appropriate size contains the correct sequence is routinely confirmed by one or more of several methods. Common approaches include Southern transfer of DNA fragments from the agarose gel combined with hybridization with specific probes (*8*), slot and dot–blot hybridization (*9*, *10*), and sandwich hybridization techniques (*11*). The probes used to identify the PCR-generated fragments in these procedures can be either radiolabeled or nonisotopically labeled (*10–12*). These procedures can also improve the sensitivity of PCR. The fragments generated by PCR may also be subjected to analysis by restriction enzymes (*13*).

PCR and its accompanying detection procedures are commonly used in a qualitative manner to determine the presence or absence of a DNA target sequence. However, PCR can also be used to quantify the amount of target nucleic acids detected (*3*). The protocols required to quantify the products of the exponential PCR process are complex and not routinely carried out.

Various applications for PCR have arisen. This procedure has been used to identify genetic disorders such as sickle-cell anemia (*1*) and cystic fibrosis (*14*). Infectious agents, including RNA and DNA viruses and microorganisms, have been diagnosed by PCR. Forensic science has applied PCR to the typing ("fingerprinting") of DNA obtained from a single hair (*15*). PCR has also become an important tool in molecular biology; it can be used to generate DNA fragments for cloning and sequencing (*7*). In addition, probes labeled with an isotope or containing a nucleotide modified with biotin (*12*) or digoxigenin (*16*) can be prepared with PCR.

Qβ Replicase Amplification

Qβ replicase is a unique RNA polymerase. The active enzyme complex of 215 kDa comprises four subunits, only one of which is encoded by Qβ bacteriophage; the remaining three subunits (S1, Tu, and Ts) are contributed by the *E. coli* host. Qβ replicase can copy only a limited family of RNA molecules derived from the Qβ bacteriophage. Lizardi et al. (*17*) were the first to adapt midivariant (MDV-1) RNAs for use as reporter molecules in hybridization assays. Midivariant-like RNA templates for Qβ replicase are highly structured RNA molecules. In part because of their structure, the daughter strands synthesized by Qβ replicase are single-stranded complements of the parent template. Each round of replication takes ~20 s at 37 °C. Because both the plus and minus strands are replicated by Qβ replicase, amplification proceeds exponentially until the number of replicates exceed the number of enzyme molecules, without the need for the high-temperature denaturation step. A single MDV-1 template can produce 10^{12} replicates in only 10–15 min at a constant temperature (37 °C).

MDV-like templates for hybridization assays have been prepared by inserting a probe sequence internal to the MDV molecule or by adding a probe sequence to the 3′-end of a MDV transcript, as described by Pritchard and Stefano (*18*). Replicating recombinant Qβ replicase probes have been constructed for *Plasmodium faciparum*, HIV-1, cytomegalovirus, *Chlamydia trachomatis*, and *Neisseria gonorrhoea* (*19*). Because Qβ replicase is used to amplify a signal molecule of a hybridization assay and because only a few molecules are required for detection, an efficient method is needed to reduce assay background. This is accomplished by reversible target capture, a process developed by Collins et al. (*20–22*).

Reversible target capture purifies specific nucleic acids through hybridization with capture probes of defined composition. The target nucleic acid is hybridized with two probes, a dA-tailed capture probe and a MDV reporter probe, in a guanidine isothiocyanate solution at 37 °C. Capture probes are typically 30–40 nucleotides long and derivatized with 100–200 residues of deoxyadenosine monophosphate by terminal deoxynucleotidyltransferase to form a poly(dA) tail (*23*). Both the capture probe and the reporter probe contain sequences complementary to the nucleic acid target, which can be RNA or DNA. If the target is double-stranded DNA, it must first be denatured by incubation at 85 °C. To apply Qβ replicase amplification, one need not convert RNA to cDNA, as must be done for PCR. After hybridization, the ternary complex formed between capture probe, reporter probe, and target is captured or bound to magnetic beads containing oligo(dT). The beads containing the nucleic acid complex are washed to remove excess unhybridized reporter probe, cell debris, and potential inhibitors. The probe–target complex is then chemically eluted from the particles by using a release buffer. This buffer selectively dissociates the dA–dT hybrids between the capture probe and the magnetic beads without disrupting the complex formed between the probes and the nucleic acid target. Used particles, which may contain nonspecifically bound MDV reporter probes or other inhibitors, are discarded after magnetic separation. This cycle of magnetic particle capture and release of the probe–target complex can be repeated as many as four times, which reduces nonspecific noise by 10^7- to 10^8-fold. About 10–30% of the target molecules present in a specimen are recovered after reversible target capture (*22*).

After the final reversible target capture step, the

hybrid complexes can be released in a low-salt buffer and the MDV reporter probes amplified without additional processing. The number of MDV reporter probes present in the eluted sample can be estimated by adding the sample to a reaction mixture containing Qβ replicase, nucleotide triphosphates, Mg^{2+}, and buffer and measuring the time required to produce a signal with an intercalating fluorescent dye. This response time is inversely proportional to the log of the number of template molecules present in the sample (*24*). The response time can be estimated by initiating multiple amplification reactions with an aliquot of the final eluate and stopping the reactions at various times by adding EDTA. The fluorescence of these end-point assays can be photographed over an ultraviolet light box or measured in a fluorometer. Alternatively, the fluorescence of the reaction mixture can be measured periodically with an automated plate reader such as the Fluoroskan-II (Flow Labs., McLean, VA) (*18*).

The specificity of assays involving reversible target capture is determined by the hybridization properties of the capture and reporter probes. This characteristic can be effected by probe design, but must be tested empirically for each application. The sensitivity of amplification assays based on Qβ replicase is limited in part by the replication characteristics of the MDV-like reporter probes.

Sample Processing

Another very important factor that affects the ultimate performance and ease of use of any nucleic acid assay is sample processing. The goal of sample processing is to lyse the cell wall of the target organism and release its nucleic acid contents. For clinical diagnostics this must be accomplished in such diverse samples as (e.g.) blood, urine, saliva, and stool. The process should also inactivate any enzymes that may degrade the nucleic acid target and remove interfering substances. Because of differences in the matrix and target organism, each sample-processing scheme must be tailored to fit its specific application. Common sample-processing schemes for PCR amplification incorporate centrifugation, proteinase K, treatments with sodium dodecyl sulfate, and phenol/chloroform extraction (*3, 5, 6, 11, 13*). For reversible target capture and Qβ replicase amplification, a chaotrope, guanidine thiocyanate, is used for sample processing (*25*). This single reagent lyses cells, dissociates macromolecular complexes, and inhibits nucleases (*26*). In addition, guanidine thiocyanate supports hybridization of the dA-tailed capture probe and the MDV-like reporter probe at 37 °C (*23*).

A single DNA target can be amplified by PCR a million-fold. Considering the requirements of sample processing, however, means that 10 to 250 DNA copies are required to produce a detectable signal by PCR (*3, 6, 8, 11*). A single MDV-1 template molecule can also be amplified by Qβ replicase to produce a detectable signal. After three cycles of reversible target capture, Pritchard and Stefano (*27*) have detected as few as 600 HIV target RNA molecules within 14 min of replication. About 2.5–3 h is required to complete the reversible target capture and Qβ replicase amplification (*27, 28*). The time to production of results for PCR varies, depending on the methods chosen by the investigator for sample processing and detection, but can range from 5 to 72 h. PCR is not routinely practiced in a manner required to produce quantitative results. The relationship between input template number and the amount of time required to produce a signal make quantification an important aspect of Qβ replicase amplification.

Both PCR and Qβ replicase are very sensitive methods. Small quantities of nucleic acid target or products produced by the amplification reactions can be sources of potential contamination. The development of a strict set of protocols is required to avoid false-positive results caused by contamination (*29*). It is wise to segregate the work areas and equipment used for sample preparation, preparation of the amplification reaction mixture, amplification, and analysis of products. Contamination can occur through contact transfer (carryover), or by aerosol generation. To minimize the physical transfer of amplifiable material during pipetting, many investigators use positive-displacement pipettes or disposable pipette tips plugged with a filter. The use of a laminar flow hood during critical transfers can reduce aerosol contamination.

By the very nature of their sensitivity, PCR and Qβ replicase amplification offer a multitude of opportunities and challenges. In time, both techniques will be reduced to relatively simple and rapid protocols. Automation will further decrease the risk of contamination and the time to results. The identification of genetic disorders or infectious agents, the typing and quantification of DNA and RNA samples, and the generation of large amounts of nucleic acid for study are but a few of the many applications for PCR and Qβ replicase.

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